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Effects of Aeration and Temperature in In Vitro and In Vivo Studies on Developing and Infective Eggs of *Ascaris suum*

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ABSTRACT: Studies were conducted on the in vitro development of eggs of *Ascaris suum* using temperatures between 20 and 32°C and continuous aeration for 46 days. The optimum temperature, defined as the temperature at which 50% of the eggs develop to the motile first larval (L1) stage in the shortest period of time was 30°C. Times for 50% development of tadpole, L1, and second larval (L2) stages were shortest at 30°C. Development appeared suboptimal at 32°C, as greater than 50% of the infective eggs showed a gradual decline in L2 motility and healthy protoplasmic appearance from 30 to 46 days in aerated cultures. Due to the inability to relate in vitro hatchability with infectivity of eggs, an in vivo system of index infectivity was established in the lungs of mice inoculated orally with 10,000 infective eggs. Eggs conditioned to growth at 30°C exhibited the greatest index of infectivity in mice at the earliest time of 21 days. The most severe index of infectivity (mortality) was obtained in mice inoculated with eggs cultured for 39 days at 26°C. There were no mortalities in mice given eggs cultured at 20 and 23°C. The rate of aeration did appear to be a differential factor in enhancing infectivity of eggs during the first 25 days of culture.

KEY WORDS: *Ascaris suum*, eggs, cultivation, temperature, aeration, mice, lung lesions.

Several authors reported that the infective larvae of *Ascaris suum* develop in decoated eggs between the temperatures of 16 and 34°C with the maximum rate of development at 31°C (Martin, 1913; Ransom and Foster, 1920; Seamster, 1950; Jaskoski, 1952; Arene, 1986). Jaskoski (1954) presented minimum and average developmental periods of 9 and 10 days at 31°C with temperatures of 31–33°C appearing to be near optimum. Timoshin (1967) reported optimal temperatures for egg development between 17 and 30°C and that the rate of development was directly proportional to temperature. Velocity curves of swine ascarid egg development at various temperatures were constructed by Seamster (1950). Cleeland and Laurence (1962) reported that decoated swine ascarid eggs were maintained at 22–26°C with frequent manual agitation until a majority of eggs had reached the infective state, usually within 30 days. Fairbairn (1961) decoated eggs and embryonated them in 0.1 N sulfuric acid at 30°C for about 20 days when they were fully infective. Rogers (1958) reported that in vitro hatching of L2 eggs could be induced following 20 days of embryonation at 30°C. Fairbairn (1961) supported this observation and stated that hatching increased linearly to near the maximum of 80% after 19 days. Arene (1986) stated that eggs embryonated at $\geqq 28 +$ 1°C had less ability to hatch and to penetrate tissue membranes in vitro than larvae from eggs embryonated at lower temperatures.

The purpose of this paper is to determine the optimum temperature for the development of eggs of *A. suum* using continuous agitation provided by slow and rapid aeration rates and various temperatures. Optimum temperatures, defined as the temperature at which 50% of the eggs would develop to the motile first larval (L1) stage in the shortest period of time, will be determined. Also, this work is intended to relate hatchability and infectivity of eggs embryonated at various conditions by experimentally infecting mice with embryonated eggs and subsequently determining mortality or lung lesion scores in mice.

Materials and Methods

Adult female *Ascaris suum* were collected from the intestines of market pigs slaughtered at a local abbatoir. The last centimeter of uteri was removed and placed in a 0.5 N NaOH solution in a Thomas tissue grinder with a grooved teflon pestle. Uteri were broken up and the eggs freed by grinding. The decoated eggs were then sterilized with a 5% peracetic acid solution for approximately 20 min. After exposure to NaOH and peracetic acid, the egg material was washed several times in sterile distilled water, each time with the aid of gentle centrifugation at 700 rpm for 5 min. The eggs were placed in 300 ml of sterile distilled water at the rate of 42,000 fertilized eggs/ml in a 500-ml Bellco dispensing funnel flask (no. 5608) tooled for rubber tubing connection at the bottom of the flask. Estimated number of eggs per flask was 12.6 million. Attached to the bottom of the funnel flask was a sterilized latex tube that was connected to an aquarium air pump. A sterile cotton filter was inserted into the air feeder tube system at the connection. Each flask had a sterilized liquid

Table 1. Time for development of eggs of *Ascaris suum* at various aeration rates and temperatures.

Temperature	BPM*	% L1 at 10 days	Mean number of days to reach stage†			
			50% tadpole	50% L1	50% L1/L2	90% L2
20	75–85	0	19.50[a]	24.00[a]	35.10[d]	38.80[f]
20	175–185	0	17.50[b]	23.00[b]	33.10[b]	40.00[d]
23	75–85	0	11.60[c]	15.00[d]	20.40[i]	33.00[i]
23	175–185	0	11.30[d]	15.00[d]	21.00[h]	40.00[d]
26	75–85	83	7.80[f]	9.30[f]	25.00[c]	38.30[h]
26	175–185	90	7.50[g]	9.00[g]	24.00[e]	37.00[j]
28	75–85	81	6.20[h]	8.80[j]	24.00[e]	41.00[b]
28	175–185	76	8.20[e]	9.80[e]	22.70[f]	41.00[b]
30	75–85	93	5.60[k]	8.25[l]	15.50[l]	24.00[k]
30	175–185	96	5.00[l]	8.50[k]	15.70[k]	20.50[l]
32	75–85	82	5.70[j]	8.80[j]	21.30[g]	39.00[c]
32	175–185	86	5.80[i]	8.80[j]	19.80[j]	36.50[g]

* BPM = number of bubbles of air per minute.

† Student–Newman–Keuls analysis ($P < 0.05$); 1-way analysis of variance. Means followed by the same letter are not significantly different at the 0.05 level.

probe thermistor connected to a telethermometer. This instrument was coupled with an automatic temperature recorder. A sterile cotton plug was then inserted in the mouth of the funnel. The flask with tubing was placed in a similar area of each environmental chamber. The aquarium air pump and telethermometer were situated outside of the chamber. A series of experiments was designed in which thermostats of 6 chambers were regulated to within ±1°C for the following temperatures: 20, 23, 26, 28, 30, and 32°C. These temperatures were selected because they were within the minimum and maximum range of 16 and 34°C reported by several authors for the development of embryonated eggs. Aeration ranges were determined by auscultation of air tubes, calculating the approximate number of sounds per minute. Ranges varied from 75 to 85 (slow aeration) and 175 to 185 (fast aeration) bubbles of air per minute. The percentage of stage of development was calculated by pipetting approximately 200 eggs from each flask in 2 equal samples taken every 2 days onto a clean microscope slide and counting the number of eggs in each stage of growth. Air was constantly introduced in the bottom of the flask during these sampling periods.

Though there were several stages in early development, i.e., single-celled, 2-celled, 4-celled, 8-celled, morula, and tadpole, the tadpole stage was chosen for the first measurement of growth. The next measurement was the percent of L1 in eggs cultured for 10 days. This time period was arbitrarily selected from growth data presented by Arene (1986), Maung (1978), and Seamster (1950). The time for these eggs of *A. suum* to reach about 50% population of L1 was the third growth assessment. To obtain the next 2 measurements, approximately 50% L1/L2 and ≧90% L2, an aliquot sample of approximately 200 eggs was taken per measurement from each culture, placed on a glass slide, and a coverslip superimposed on the egg sample. Pressure was applied to the coverslip, which liberated larvae from the eggs. According to Maung (1978), L1 larvae are so delicate that they could not withstand being pressed. After the first molt, the L2 larvae were able to withstand pressure. In addition, the L1 cuticle separated from the L2 until a loose sheath was formed around the L2. Eventually the L1 cuticle was lost inside the egg, so that when pressure was applied a naked L2 appeared. L1 and L2 larvae were differentiated and counted using the guidelines of Maung (1978).

To conduct infectivity studies, Cox White Swiss mice, weighing approximately 20 g each, were allotted to groups of 6 mice each. The number of animal groups per experiment was 4. An oral dose of approximately 10,000 embryonated eggs of *A. suum* per mouse was given by intubation (Boisvenue et al., 1968) at periodic times during development. Ten days after infection, all mice were killed in a carbon dioxide gas chamber, and the excised lungs were immediately examined and scored according to Brown and Chan (1955). The degree of lung damage caused by migrating L2 was classified based on the extent of hemorrhage and consolidation and scored from 0 to 5. Lungs apparently normal were scored 0; complete hemorrhage of both lungs was scored 5. Mean lung lesion scores were determined for each group. Infectivity of eggs was measured by the number of rodent deaths, the range of individual scores, and the group mean score of 6 mice. Statistical analysis was conducted using Student–Newman–Keuls method ($P < 0.05$), 1-way analysis of variance.

Results

The greatest percentage of L1 stages observed at 10 days was in cultures at 30°C, followed closely by those eggs cultivated at 26°C (Table 1). There were no L1 stages seen at this time in cultures set at 20 and 23°C. The temperature at which swine ascarid eggs developed to a 50% motile L1 population in the shortest period, i.e., optimum

Table 2. Mean lung lesion scores of groups of mice experimentally infected with 10,000 eggs of *Ascaris suum* cultured at 20 and 26°C.

Age of cultures (days)	Group no.	Temp. °C	Aeration (BPM)*	Mean group score†	Range of scores	Mortalities
25	1	20	75–85	0.00[d]	(0)	0
	2	26	75–85	2.00[a]	(0)	0
	3	20	175–185	0.71[c]	(0–1)	0
	4	26	175–185	1.14[b]	(1–2)	0
31	1	20	75–85	1.00[c]	(0)	0
	2	26	75–85	2.71[b]	(1–4)	1
	3	20	175–185	1.00[c]	(0)	0
	4	26	175–185	4.14[a]	(2–5)	3
39	1	20	75–85	1.00[c]	(0)	0
	2	26	75–85	2.00[b]	(1–4)	2
	3	20	175–185	0.00[d]	(0)	0
	4	26	175–185	4.16[a]	(3–5)	4
46	1	20	75–85	0.33[c]	(0–1)	0
	2	26	75–85	1.50[a]	(1–4)	1
	3	20	175–185	1.00[b]	(0)	0
	4	26	175–185	1.33[a]	(1–3)	1

* BPM = bubbles per minute.
† Student–Newman–Keuls analysis ($P < 0.05$).

time, was 30°C. In addition, this temperature promoted the greatest development in tadpoles, 50% L1/L2, and 90% L2 stages (Table 1). In general, it appeared that a faster aeration rate improved the development of tadpoles and 50% L1 at temperatures from 20 to 26°C.

Mean lung lesion scores determined from groups of mice experimentally infected with embryonated eggs cultured at various temperatures, time, and aeration are presented in Tables 2–4. Scores were very low in mice infected with eggs cultured for 25 days at 20°C (Table 2). Infectivity of these eggs was generally poor following 31, 39, and 46 days cultivation. Eggs developed at 26°C were determined to be more infective in mice inoculated at these times. Twenty-five to 58% mortalities were observed in mice given eggs cultured for 31 and 39 days. Rapid aeration of cultures appeared to enhance infectivity of eggs grown at 26°C during this high mortality period, but not at 46 days. Mice in this group were not infected with eggs cultured for 21 days.

In the second experimental group (Table 3), eggs cultured at 23°C for 21 and 25 days were noninfective. However, as culturing continued, rapid aeration generally improved infectivity of these eggs at this temperature. Eggs cultured at 30°C were more infective in mice in the initial growth period, i.e., 21, 25, and 32 days. There appears to be some difference in parasitic infectivity in mice due to a difference in aeration of eggs set at 30°C. With aging to 39 and 46 days, eggs exposed to 23°C had similar infectivity (lesion scores) in mice as those conditioned at 30°C.

Infectivity was more pronounced at 21 days in mice infected with eggs cultured at 28 and 32°C (Table 4). However, at 31 days of continuous growth, the infectivity of eggs set at 32°C diminished. This condition was corroborated by microscopic observation of embryos that were gradually deteriorating to a poor protoplasmic appearance with little L2 motility. Eggs maintained in the 28°C environment retained a good level of infectivity throughout. The rate of aeration did appear to be a differential factor in enhancing infectivity of these eggs during the first 25 days of culture.

Discussion

The lower percentage of L1 found in eggs after 10 days in culture (DIC) at 28°C, especially at the higher aeration rate, cannot be explained by a difference in pooled egg material (Table 1). However, it was observed microscopically at 12 days that the L1 percentages in both aerated cultures were at least equal to those percentages in eggs set at 26°C. In general, the mean number of days at the temperature required to produce 50% L1 was significantly less in the present study compared to Seamster's (1950) study (Table 5).

Table 3. Mean lung lesion scores of groups of mice experimentally infected with 10,000 eggs of *Ascaris suum* cultured at 23 and 30°C.

Age of cultures (days)	Group no.	Temp. °C	Aeration (BPM)*	Mean group score†	Range of scores	Mortalities
21	1	23	75–85	0.0[d]	(0)	0
	2	30	75–85	2.8[b]	(2–5)	2
	3	23	175–185	0.0[d]	(0)	0
	4	30	175–185	2.8[b]	(2–4)	1
25	1	23	75–85	0.0[d]	(0)	0
	2	30	75–85	2.66[a]	(2–3)	0
	3	23	175–185	0.0[d]	(0)	0
	4	30	175–185	2.50[a]	(2–3)	0
31	1	23	75–85	1.50[b]	(1–2)	0
	2	30	75–85	2.50[a]	(2–4)	1
	3	23	175–185	1.00[c]	(0–2)	0
	4	30	175–185	2.11[a]	(2–3)	1
39	1	23	75–85	1.66[b]	(1–3)	0
	2	30	75–85	1.83[b]	(1–2)	0
	3	23	175–185	2.00[b]	(0)	0
	4	30	175–185	2.66[a]	(2–3)	0
46	1	23	75–85	1.83[d]	(1–2)	0
	2	30	75–85	2.16[b]	(2–3)	0
	3	23	175–185	2.50[a]	(2–3)	0
	4	30	175–185	1.83[d]	(0–3)	1

* BPM = bubbles per minute.

† Student–Newman–Keuls analysis ($P < 0.05$).

Table 4. Mean lung lesion scores of groups of mice experimentally infected with 10,000 eggs of *Ascaris suum* cultured at 28 and 32°C.

Age of cultures (days)	Group no.	Temp. °C	Aeration (BPM)*	Mean group score†	Range of scores	Mortalities
21	1	28	75–85	2.60[b]	(2–3)	1
	2	32	75–85	2.16[d]	(2–3)	0
	3	28	175–185	2.20[c]	(1–3)	1
	4	32	175–185	2.75[a]	(1–5)	2
25	1	28	75–85	2.16[b]	(1–4)	2
	2	32	75–85	1.33[d]	(1–2)	0
	3	28	175–185	1.83[c]	(1–2)	0
	4	32	175–185	2.33[a]	(1–3)	0
31	1	28	75–85	2.50[a]	(2–3)	0
	2	32	75–85	1.00[c]	(0)	0
	3	28	175–185	2.33[a]	(2–3)	0
	4	32	175–185	1.16[b]	(1–2)	0
39	1	28	75–85	1.66[a]	(1–3)	0
	2	32	75–85	0.83[c]	(0–1)	0
	3	28	175–185	1.83[a]	(1–3)	0
	4	32	175–185	1.00[b]	(0–2)	0
46	1	28	75–85	2.33[a]	(2–3)	0
	2	32	75–85	1.33[c]	(1–2)	0
	3	28	175–185	2.00[b]	(1–3)	0
	4	32	175–185	1.20[d]	(1–2)	0

* BPM = bubbles per minutes.

† Student–Newman–Keuls analysis ($P < 0.05$).

Table 5. Time required for development of eggs of *Ascaris suum* to reach 50% motile L1 at various temperatures.

	Mean number of days at temperature (°C)					
	20	23	26	28	30	32
Seamster (1950)	19.17[a]*	12.96[c]	12.95[d]	9.71[f]	8.88[g]	8.75[h]
This study†	18.50[b]	11.45[e]	7.65[i]	7.20[j]	5.30[l]	5.75[k]

* Student–Newman–Keuls analysis ($P < 0.05$).
† Mean of slow and fast aeration rates

This difference was greatly significant at ≧26°C and is attributed to the continuous agitation of eggs by means of aeration, which was not conducted in the 1950 experiment. The earlier study involved humidity requirements for culturing that were not necessary in the present study. Again, eggs cultured at 30°C required the least amount of time for development.

There appear to be conflicting reports about the sequence of events occurring during the second molt in the development of *A. suum* larvae. Roberts (1934) stated that the second molt was in the lungs, and Douvres et al. (1969) reported that this molt occurred in the liver. Araujo (1972) and Maung (1978) related that the early stages of development in the egg comprised 2 molts, each occurring soon after the other. However, the time of completion of the second molt varied considerably, which sometimes was not completed until the larvae reached the liver (Maung, 1978). The author of this report presents the case that the larval stage identified in the infective egg (Table 1) is the L2 stage and makes no attempt to present larval types A–I as did Maung (1978). L1 and L2 stages were identified by their ability to withstand hatching by the coverslip pressure method and by the presence of the L1 cuticle loose at the anterior and posterior ends, around the body of the liberated L2.

Due to the inability to relate in vitro hatchability with infectivity of eggs, an in vivo system in mice was established. On the basis of lung lesion scores in mice, it was possible to identify egg samples cultured at lower temperatures of 20 and 23°C. Eggs conditioned to growth at 30°C exhibited the greatest index of infectivity at the earliest time of 21 days. The most severe index of infectivity (i.e., mortality) was attained in mice inoculated with eggs cultured for 31 and 39 days at 26°C (Table 6). Arene (1986) stated that eggs embryonated at ≧28°C had less ability to hatch and to penetrate tissue membranes in vitro than larvae from eggs embryonated at lower temperatures. In the present study, there were no mortalities in mice infected with eggs cultured at 20 and 23°C regardless of time. However, the infectivity rates indicated by mortalities were higher in mice inoculated with eggs in the early days of culture at ≧28°C. Cultivation temperatures of 28 and 32°C were correlated with points of infectivity. The latter temperature is interpreted as approaching the maximum tolerated temperature, as L2 larvae in the eggs were observed microscopically to show a gradual decline in larval motility and in healthy protoplasmic appearance at 30–46 days of cultivation. This interpretation approximates that given by Seamster (1950) and was verified by lower lung lesion scores obtained

Table 6. Mean lung lesion scores of groups of mice inoculated orally with 10,000 eggs of *Ascaris suum* cultured at different temperatures.

	Temperature (°C)											
	20†		23		26		28		30		32	
DIC*	S	F	S	F	S	F	S	F	S	F	S	F
21	N/D	N/D	0.00[h]*	0.00[h]	N/D	N/D	2.60[d]	2.20[e]	2.80[b]	2.80[b]	2.16[f]	2.75[c]
25	0.00[l]	0.71[i]	0.00[l]	0.00[l]	2.00[d]	1.14[h]	1.75[f]	1.83[e]	2.66[a]	2.50[b]	1.33[g]	2.33[c]
31	1.00[l]	1.00[l]	1.50[g]	1.00[l]	2.71[b]	4.14[a]	2.50[d]	2.33[e]	2.50[d]	2.11[f]	1.00[l]	1.16[h]
39	1.00[j]	0.00[l]	1.66[g]	2.00[c]	1.00[j]	4.16[a]	1.66[g]	1.83[e]	1.83[e]	2.66[b]	0.83[k]	1.00[j]
46	0.33[l]	1.00[k]	1.83[f]	2.50[a]	1.00[k]	1.00[k]	2.33[b]	2.00[d]	2.16[c]	1.83[f]	1.33[g]	1.20[h]

* DIC = days in culture.
† S/F = slow and fast aeration; N/D = not done; Student–Newman–Keuls analysis ($P < 0.05$).

in mice inoculated with eggs set at 32°C for 31, 39, and 46 days.

The present study suggests that the aeration rate and temperature at which eggs of *A. suum* are embryonated have an effect on the viability, hatchability, and subsequent infectivity of L2 larvae in mice.

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1990 Student Presentation Competition

The October 1990 meeting of the Society will be devoted to the second student research presentation competition, which will be held at the Uniformed Services University of the Health Sciences. Cash awards will again be made for the best paper(s). A call for papers will appear in the July issue of the *Journal.* For more information, contact Dr. Patrick Carney, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814.